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(51) International Patent Classification: C12Q 1/68, (74) Agent: TUSCAN, Michael S.; Morgan, Lewis & Bockius LLP, 1800 M. Street, N.W., Washington, DC 20036-5859 (US).

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(71) Applicant (for all designated States except US): GENE LOGIC, INC. (USUS); 708 Quince Orchard Road, Gaithersburg, MD 20878 (US).

(72) Inventors and (73) Inventor/Applicant (for US only): EINSTEIN, Richard (USUS); 708 Quince Orchard Road, Gaithersburg, MD 20878 (US).

(54) Title: MODULATION OF EGR-1 AND EGR-2 IN HEART DISEASE

(57) Abstract: Methods are described for diagnosing coronary artery disease associated with ischemia by measuring the induction of early growth response factor-1 (Egr-1) and early growth response factor-2 (Egr-2) genes in nucleic acid samples from patients. Also described are methods of screening modulators of Egr-1 and/or Egr-2 induction. Further, forensic methods are described for determining ischemic related pathology. The figure 1A depicts an image of a READS gel demonstrating up regulation of human Egr-1 between RNA samples obtained from age and sex matched controls (first two lanes) versus heart failure patients (last five lanes).

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(78) Sequence: 1084 Q-PCR Results

(79) Sequence: 1084 Q-PCR Results

MODULATION OF EGR-1 AND EGR-2 IN HEART DISEASE

FIELD OF THE INVENTION

This invention relates to methods which monitor the induction of expression of the immediate early genes (IEGs), early growth response gene-1 (Egr-1) and early growth response gene-2 (Egr-2). Such monitoring can serve 1) as a predictor of the effects of post ischemic events, such as heart attack and myocardial infarction, 2) as a marker to elucidate pathology of diseases related to coronary artery disease (CAD) such as sudden cardiac death (SCD) or 3) as a means of identifying agents that modulate the induction of these IEGs in cardiac tissues. This application claims priority to U.S. Provisional Application No. 60/142,973, filed July 12, 1999, which is herein incorporated by reference in its entirety.

BACKGROUND

Cardiovascular disease (CVD), principally heart disease and stroke, is the Nation's leading cause of death for both men and women among all racial and ethnic groups. More than 960,000 Americans die of CVD each year, accounting for 42% of all deaths.

One in four Americans have CVD. Heart disease and stroke account for almost 6 million hospitalizations each year and cause disability for almost 10 million Americans aged 65 years and older. CVD costs the nation \$274 billion each year, including health expenditures and lost productivity.

Most heart attacks and strokes are caused by coronary artery disease (CAD). During this process, ischemia occurs when the blood supply to part of the heart muscle itself is severely reduced or stopped. This occurs when one of the arteries that supply blood to the heart muscle (coronary arteries) is blocked by an obstruction, usually caused by the buildup of plaque along the vessel walls, i.e., atherosclerosis. When the blood supply is cut off, muscle cells suffer irreversible injury and die. Consequently, disability or death can result, depending on how much heart muscle is damaged.

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Genes which are involved in cardioprotection and/or repair processes may be identified by methods which result in the isolation of differentially expressed genes.

Several groups have identified differentially expressed genes associated with cardiac conditions, such as cardiac hypertrophy (Masuda *et al.*, *FEBS Lett* (1997) 406(2):221-4), in developing rat heart (Comier-Regard *et al.*, *Mol Cell Biochem* (1997) 172(1-2):111-20), cardiotoxic doxorubicin effects (Teyssie *et al.*, *J Biol Chem* (1997) 272(6):22800-8), stretch-induced cardiac fibroblast stress (Tyagi *et al.*, *J Cell Physiol* (1998) 176(2):374-82), as well as left ventricular pressure overload hypertrophy and decompensated congestive heart failure (Collins *et al.*, *J Mol Cell Cardiol* (1996) 28(7):1435-43). However, differential display techniques have not been employed to characterize coronary artery disease or the effects of ischemia on heart muscle.

Egr-1 and Egr-2

Expression of Egr-1 is induced by a wide range of stimuli in diverse cell types (Decker *et al.*, *J Biol Chem* (1998) 273(41):26923-26930) and has been associated with myocardial protein synthesis (Neyes *et al.*, *Biochem Biophys Res Comm* (1991) 181(1):22-27) and cardiac remodeling (Sharma *et al.*, *Biochem Biophys Res Comm* (1994) 205(1):105-112). For example, mechanical stress such as passive stretch of cardiomyocytes activates protein synthesis and particularly induces the expression of Egr-1 (Yamaguchi *et al.*, *J Mol Cell Cardiol* (1995) 27(1):133-140). Unlike skeletal muscle cells in which growth and differentiation appear mutually exclusive, growth stimulation of cardiac cells is characterized by transient expression of immediate early genes (proto-oncogenes) as well as induction of several cardiac specific markers (Macdonald *et al.*, *Mol Cell Biol* (1993) 13(1):600-612). Moreover, because hemodynamic load (e.g., blood pressure) is a primary regulator of cardiac mass, a proximal event in the regulatory pathway for this increase in cardiac mass is the induction of immediate early genes such as fos, jun and Egr-1 in response to a significant increase in blood pressure (Rozich *et al.*, *J Mol Cardiol* (1995) 27(1):485-499).

Like Egr-1, Egr-2 induction by various stimuli has been demonstrated in a number of diverse tissues (Beckman *et al.*, *Neurochem Int* (1997) 31(4):477-510). While fibroblasts

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and immune cells dominate much of the scientific literature for *in vitro* characterization of the general mechanism of Egr-2 induction (Newton *et al.*, *Eur J Immunol* (1996) 26(4):811-816; Gotschalk *et al.*, *J Immunol* (1994) 152(5):2115-2122; Miltenberger *et al.*, *J Biol Chem* (1993) 268(21):15674-15680; and Uchizono *et al.*, *Cell Struct Funct* (1994) 19(5):341-348), many of the *in vivo* studies have focused on the brain (Hendegen *et al.*, *Brain Res Brain Res Rev* (1998) 28(3):370-490; Beckman *et al.*, *Neurochem Int* (1997) 31(4):477-510; and Hendegen *et al.*, *Neuroscience* (1993) 57(1):41-52). This includes drug effects on the brain (Nakdi *et al.*, *J Neurosci Res* (1996) 45(1):13-27; and Lebrun *et al.*, *Neuroscience* (1995) 65(1):93-99), and brain injury paradigms, such as viral infection of neurons (Tatarowicz *et al.*, *J Neurovirol* (1997) 3(3):212-224). However, the association between Egr-2 expression and heart or heart stressors has not been identified before the invention described herein.

Much of the scientific literature discloses the simultaneous analysis of both Egr-1 and Egr-2 expression (e.g., Beckman *et al.*, *Neurochem Int* (1997) 31(4):477-510), although their expression is often diametric. For example, this diametric expression has been observed in HSV-1 latency transcription induction (Tatarowicz *et al.*, *J Neurovirol* (1997) 3(3):212-224), angiotensin II exposure induction in the brains of rats (Lebrun *et al.*, *Neuroscience* (1995) 65(1):93-99), estradiol effects in the rat uterus (Cicalillo *et al.*, *Receptor* (1993) 3(1):17-30) and within specific cell lines (Gotschalk *et al.*, *Eur J Immunol* (1993) 23(6):2011-2015).

In the present invention, Egr-1 and Egr-2 have been observed to be induced in nucleic acid samples from ischemic heart tissues. Demonstration of such induction in nucleic acid samples obtained from living patients can serve as a diagnostic aid for detection of coronary artery disease. Also, such a demonstration of induction can serve as a means of elucidating CAD pathology in postmortem tissues. Moreover, cells in which Egr-1 and/or Egr-2 can be induced can be used to screen compounds which modulate the expression of these factors.

SUMMARY OF THE INVENTION

According to the present invention, transcription factors, Egr-1 and Egr-2 have been observed to be induced in tissues isolated from human heart under conditions of ischemia.

In a preferred embodiment, Egr-1 or Egr-2 are detected in a method of diagnosing coronary artery disease (CAD) in a patient, wherein nucleic acid samples are used to assay for the induction of Egr-1 or Egr-2. In a further embodiment, a screening method is contemplated for identifying modulators of Egr-1 or Egr-2 induction.

In another preferred embodiment, a method of determining a cause of death where coronary artery disease is suspected is contemplated wherein nucleic acid samples are screened for the induction of Egr-1 or Egr-2. In a related aspect, the induction of Egr-1 or Egr-2 is indicative of CAD as the cause of death.

This and other aspects of the present invention will become clearly understood by reference to the following figures, examples and description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A, B and C: Quantitative PCR (Q-PCR) Results for Egr-1. In Figure 1A an image of a READS gel demonstrating up regulation of human Egr-1 between RNA samples obtained from age and sex matched controls (first two lanes) versus heart failure patients (last five lanes). The single headed arrow identifies the band of interest. In Figure 1B, the bar graph demonstrates, graphically, differential expression of Egr-1 in healthy human control samples versus ischemic heart samples (Cluster 1) and samples from patients exhibiting idiopathic heart disease (Cluster 2). The ordinate represents fold difference between the amount of Egr-1 measured versus internal control using quantitative PCR and the abscissa is the sample identifier. In Figure 1C, the bar graph demonstrates, graphically, differential expression of Egr-1 in healthy human control samples versus heart failure disease panel. The heart failure disease panel consists of samples from human failing hearts that were removed for transplant. Each sample has clinical data and basic physiology that was performed on isolated myocytes. The samples represent a range of failing cells from those that could be barely be considered different from normal cells to those that are severely compromised in their function. Cluster 1 contains samples that are categorized as ischemic heart failure patients. These patients had some episode that allowed the assessment that the patient had undergone some event that reduced blood flow to the heart. Cluster 2 contains samples from

patients classified as presenting idiopathic heart failure. These patients have no clear assessment as to the impairment of heart function, but ischemia has been ruled out through laboratory test. The heart failure panel contains a mixture of ischemic, idiopathic, valvular, i.e., patients with damaged valves) and other diagnoses of disease. Again, these samples have a range of function from fairly healthy to extreme disease. The ordinate represents fold difference between the amount of Egr-1 measured versus internal control using quantitative PCR and the abscissa is the sample identifier.

Figure 2A and B: Quantitative PCR (Q-PCR) Results for Egr-2. In Figure 2A, the bar graph demonstrates, graphically, differential expression of Egr-2 in healthy human control samples versus ischemic heart samples (Cluster 1) and samples from patients exhibiting idiopathic heart disease (Cluster 2). The ordinate represents fold difference between the amount of Egr-2 measured versus internal control using quantitative PCR and the abscissa is the sample identifier. In Figure 2B, the bar graph demonstrates, graphically, differential expression of Egr-2 in healthy human control samples versus heart failure disease panel. The ordinate represents fold difference between the amount of Egr-2 measured versus internal control using quantitative PCR and the abscissa is the sample identifier.

Figure 3: Graphic representation of the distribution of Egr-1 expression in various human tissues. RT-PCR is performed on nucleic acids isolated from the human tissues listed. After normalizing data to GAPDH expression, relative expression is determined by comparing the amount of Egr-1 PCR product in any given tissue to that measured human brain.

Figure 4: Graphic representation of the distribution of Egr-2 expression in various human tissues. RT-PCR is performed on nucleic acids isolated from the human tissues listed. After normalizing data to GAPDH expression, relative expression is determined by comparing the amount of Egr-2 PCR product in any given tissue to that measured human brain.

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Figure 5: Agarose gel of the distribution of Egr-1 expression in various human tissues determined by RT-PCR.

DETAILED DESCRIPTION OF THE INVENTION

General Description

The present invention is based in part on identifying genes that are differentially regulated or expressed in human ischemic heart tissue compared to normal heart tissue. These genes, as well as the peptides they encode, can serve as targets for agents that can be used to inhibit the expression or modulate the activity of the polypeptides they encode. For example, agents may be identified which modulate biological processes associated with ischemic injury to the heart such as chronic ischemic heart disease and ischemic cardiomyopathy. Agents may also be identified which modulate the biological processes associated with recovery from ischemic injury to the heart.

Definitions

"Coronary artery disease" (CAD) herein refers to a pathological condition of the heart caused by occlusion of blood vessels, resulting in the interference of the flow of blood to different segments of the myocardium. Such interference can result in angina pectoris, myocardial infarction and sudden cardiac death. For example, angina pectoris is a syndrome due to myocardial ischemia characterized by episodes of chest discomfort or pressure. In a further aspect, myocardial infarction herein refers to necrosis of myocardium due to ischemia resulting from abrupt reduction in coronary blood flow to segments of the heart. In a further related aspect, sudden cardiac death (SCD) is a swift and unexpected death which occurs within moments of the onset of acute symptoms (e.g., chest pain).

"Chronic diffuse myocardial ischemia" herein refers to a disease wherein a patient exhibits persistent and recurrent reduction in blood flow to segments of the myocardium due to blood vessel obstructions which are accompanied by distinctive deviations in wave forms in data from electrocardiograms (ECG).

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"Myocardium" herein refers to the muscle tissue of the heart.

"Ischemia" herein refers to a decrease in blood supply to a body organ, tissue or part caused by constriction or obstruction of blood vessels.

"Shear stress" herein refers to the energy necessary produce an opposite but parallel sliding motion across a body's plane. In a related aspect, "shear stress" refers to substantially the physiological equivalent pressure produced in various tissues or organs such as force present in the vasculature by the actions of cardiac muscle.

"Modulate" as used herein refers to the inhibition, induction, agonism and/or antagonism of the regulation of expression or regulation of function of Egr-1 and/or Egr-2 genes or gene products.

"Nucleic acid" includes DNA and RNA molecules and is used synonymously with the terms "nucleic acid sequence" and "polynucleotide."

"Transcription factors" herein refers to a class of proteins that bind to a promoter or to a nearby sequence of DNA to facilitate or prevent transcription initiation.

"Polypeptide" herein refers to an amino acid sequence including, but not limited to, proteins and protein fragments, naturally derived or synthetically produced.

"Transcriptional profiling" herein refers to any assay method or technique which is capable of selectively analyzing, quantitatively and/or qualitatively, one or more mRNA species found in a cell or a nucleic acid sample. For example, such assays include but are not limited to RT-PCR, quantitative PCR (Q-PCR), RNase protection assays, and Northern blots.

"Zinc finger transcription factor" or "zinc finger domain" herein refers to a protein having a zinc binding domain comprising a Cys-Xaa-Cys-Xaa-Cys-Xaa-Cys (or His) or similar sequence that provides the opportunity of Zn(II) binding.

Preferred Embodiments

Egr-1 and Egr-2 are known inducible transcription factors (ITFs) which, as a consequence of their induction, modulate the expression of their target genes thereby altering a tissues' responses to subsequent stimuli. Two attributes characteristic of these ITFs are (i) their rapid expression, which is controlled by pre-existing transcription factors and (ii) their functioning as transcription factors. For example, Egr-1 usually functions as an activator of

target gene transcription such as for rat phenylethanolamine N-methyl transferase, human apolipoprotein A1, and platelet derived growth factor B chain genes. On the other hand, Egr-1 can also repress expression (e.g., repression of murine adenosine deaminase). As a further example, Egr-2 has been observed to transactivate HoxB2, the insulin-like growth factor II and HoxA4 genes.

Egr-1 and Egr-2 are characterized as zinc finger transcription factors of the Cys2His2 class (Beckman *et al.*, *Neurochem Int* (1997) 31(4):477-510). These proteins share extensive homology (90% identity or conserved residues) throughout a zinc finger DNA-binding domain and recognize the same consensus DNA binding motif.

As used herein, homology or identity can be determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin *et al.*, *Proc Natl Acad Sci USA* 87: 2264-2268 (1990) and Altschul, S. F. *J Mol Evol* 36: 290-300(1993), fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (*Nature Genetics* 6: 119-129 (1994)) which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (i.e., the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff, *et al.*, *Proc Natl Acad Sci USA* 89: 10915-10919 (1992), fully incorporated by reference). For blastn, the scoring matrix is set by the ratios of M (i.e., the reward score for a pair of matching residues) to N (i.e., the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively.

Egr-1 and Egr-2 recognize the consensus motif GCG(G/T)GGGCG which is commonly referred to as the GSG motif (Herdegen T., *Neurochem Int* (1997) 31(4): 517-6). In a related aspect, the consensus binding motif for SP1, which is related to and often

overlaps the Egr-1 or Egr-2 consensus binding site, does not compete for binding to the Egr-1 or Egr-2 protein.

In a preferred embodiment, Egr-1 DNA comprises the nucleic acid sequence as set forth in SEQ ID NO: 1, wherein said DNA encodes the amino acid sequence as set forth in SEQ ID NO: 1, wherein said DNA encodes the amino acid sequence as set forth in SEQ ID NO: 2.

In a further preferred embodiment, Egr-2 DNA comprises the nucleic acid sequence as set forth in SEQ ID NO: 3, wherein said DNA encodes the amino acid sequence as set forth in SEQ ID NO: 4.

As used herein, "Egr-1 DNA" and "Egr-2 DNA" are not limited to the sequences

defined by SEQ ID NOS: 1-4, and include those nucleic acid sequences which are related to SEQ ID Nos: 1 and 3 or which encode these transcription factors in other mammals, for example, those nucleic acids which encode Egr-1 and Egr-2 in rat and murine species. See Cao *et al.*, *J Biol Chem* (1992) 267(18):12991-12997; Cicciello *et al.*, *Receptor* (1993) 3(1):17-30; and Lebrun *et al.*, *Neuroscience* (1995) 65(1):93-99, herein fully incorporated by reference, or, for example porcine sequences are described by Brand *et al.*, *Circ Res* (1992) 71(6):1351-60.

In order to assay Egr-1 or Egr-2 expression of the present invention in a physiologically relevant manner, tissues may be assayed under conditions which model physiological cardiac cell stimuli. For example, some model systems simply include substrate depletion and increased intracellular acidity (Ch'en *et al.*, *Prog Biophys Mol Biol* (1998) 69(2-3):515-38). Others are more complex. For example, Wilders *et al.* used isolated guinea pig ventricular myocytes which were electrically coupled via a coupling-clamp circuit to a comprehensive computer model of a guinea pig ventricular myocyte to assess alterations in the critical value of coupling conductance required for action potential conduction from the real cells to the model cell when the real cells are exposed to a solution that simulates acute ischemia (Wilders *et al.*, *Circulation* (1999) 30:99(12):1623-9). Further, exposure of myocytes to tumor necrosis factor- α , IL-1 β , or lipopolysaccharide has been shown to simulate effects seen during reperfusion injury (Gwechenberger *et al.*, *Circulation* (1999) 99(4):546-51). Further, models have been developed to simulate ischemia and reperfusion in quiescent human ventricular cardiomyocytes. Cellular injury and metabolic parameters can be

assessed after various interventions, such as: preconditioning cells with anoxia, hypoxia, anoxic supernatants, or hypoxic supernatants (Cohen *et al.*, *Circulation* (1998) 98(19 Suppl):II184-94; discussion II194-6). Another model is hypoxia-reoxygenation stress in the rat myoblast cell line, H9c2, which simulates ischemic preconditioning in heart tissue (Sakamoto *et al.*, *Biochem Biophys Res Commun* (1998) 20:251(2):576-9).

In a preferred embodiment, assays which incubate cells under conditions that simulate cardiac ischemia and/or heart stress *in vitro* include, but are not limited to, for example, fluid shear stress in human endothelial cells (Houston *et al.*, *Arterioscler Thromb Vasc Biol* (1999) 19(2):281-289) and passive stretch of cultured myocytes (Yamazaki *et al.*, *J Mol Cell Cardiol* (1995) 27(1):133-140). In a related aspect, assays which simulate ischemia by stressing the heart *in vivo* include, but are not limited to, for example, occlusion of the heart by ligation of blood vessels in animal models (Solomon *et al.*, *J Am Coll Cardiol* (1999) 33(3):854-856 and Kijima *et al.*, *Jpn Circ J* (1998) 62(4):294-298).

One means of diagnosing CAD using the transcriptional factors of the present invention

invention involves obtaining heart tissue from living subjects. Obtaining tissue samples from living sources is problematic for tissues such as heart. However, due to the nature of the treatment paradigms for cardiac patients, biopsy has become routine for certain procedures. For example, such procedures are used to confirm noninvasive findings at cardiac catheterization (e.g., BEG, Salem *et al.*, *New Engl J Med* (1990) 323:1261-1270). Further, biopsy of cardiac material is routinely performed in order to evaluate lymphocyte infiltration and interstitial edema post-catheterization during IL-2 administration (Beck *et al.*, *Wisc J Med* (1991) 155(3):293). Further, the procedure is common before and after cardiac transplantation (Gout *et al.*, *N Engl J Med* (1990) 322:383-388) and when secondary heart disease due to carcinoma is suspected (Rutherford *et al.*, *N Engl J Med* (1992) 327:1442-1448).

In a preferred embodiment, cardiac cells are obtained from biopsy specimens of patients where the biopsy procedure is used to confirm noninvasive findings. In a further preferred embodiment, cardiac cells are obtained from biopsy specimens of patients undergoing catheterization after a post-ischemic event (e.g., myocardial infarction). In a further preferred embodiment, cardiac cells are obtained from a patient before and after

cardiac transplantation. Biopsy can be accomplished by any means, which includes but is not limited to, those described in Giuntino *et al.* (U.S. Patent No. 5,615,690), Vesely *et al.* (U.S. Patent No. 5,797,849) or Crosby (U.S. Patent No. 4,319,562).

The use of molecular/biological tools has become routine in forensic technology. For example, nucleic acid probes may be used to determine the expression of Egr-1 and Egr-2 in an assay for CAD in forensic/pathology specimens. Further, nucleic acid assays may be carried out by any means of conducting a transcriptional profiling analysis. In addition to nucleic acid analysis, forensic methods of the invention may target the proteins encoded by Egr-1 or Egr-2 nucleic acids to determine up or down regulation of the genes. Egr-1 and Egr-2 proteins can be isolated from cardiac tissues and analyzed appropriately (Shivrick *et al.*, *Biochim Biophys Acta* (1975) 393(1):124-33).

In a related aspect, as muscle tissue is very fibrous and requires extensive manipulation (e.g., grinding in a blender), methods of the invention may involve treatment of tissues with collagenases or other proteases to make the tissue amenable to cell lysis

(Semenov *et al.*, *Bull Exp Biol Med* (1987) 104(7):13-6). Further, it is possible to obtain different regions of the heart for analysis, especially the left ventricle (see McEwen *et al.*, *J Cardiovasc Pharmacol* (1998) 31 Suppl 1:S443-6).

In a preferred embodiment, all assays will be carried-out with appropriate controls.

Methods to Identify Agents that Modulate the Expression of a Nucleic Acid

Another embodiment of the present invention provides methods for identifying agents that modulate the expression of Egr-1 and/or Egr-2. Such assays may utilize any available means of monitoring for changes in the expression level of Egr-1 and/or Egr-2 mRNA. As used herein, an agent is said to modulate the expression of Egr-1 and/or Egr-2 mRNA, if it is capable of up- or down-regulating expression of an appropriate nucleic acid in a cell.

In one assay format, cell lines that contain reporter gene in-frame fusions between the Egr-1 or Egr-2 promoter (SEQ ID NO: 5 or SEQ ID NO: 6, respectively, see Sakamoto *et al.*, *Oncogene* (1991) 6(5):867-71 and Joseph *et al.*, *Proc Natl Acad Sci U S A* (1988) 85(19):7164-8, for promoter sequences in humans) and any assayable fusion partner may be

prepared. For example human promoters as described in Sakamoto *et al.* (*ibid*) and Joseph *et al.* (*ibid*) could be used to make such fusion constructs.

Constructs containing downstream transactivation targets for Egr-1 or Egr-2 may be used to identify agents which modulate the expression of the transcription factors. For example, Egr-1 is known to repress murine adenosine deaminase upon induction (Herdegen T., *Neurochem Int* (1997) 31(4): 517-6), therefore, agents which negatively modulate Egr-1 expression may be screened with murine adenosine deaminase comprising constructs by monitoring the expression of murine adenosine deaminase in appropriately transformed cells. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Alam *et al.* (1990) *Anal. Biochem.* 188:245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time period. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents which modulate the expression of an Egr-1 or Egr-2 nucleic acid.

Additional assay formats may be used to monitor the ability of the agent to modulate the expression of a nucleic acid encoding a protein of the invention, for example, the protein having SEQ ID NO: 2 or SEQ ID NO: 4. For instance, mRNA expression may be monitored directly by hybridization to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time periods, after which total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, 1989), and followed by the appropriate hybridization analysis for example Northern blots (*ibid*).

Probes to detect differences in RNA expression levels between cells exposed to the agent and control cells may be prepared from Egr-1 and Egr-2 nucleic acids. It is preferable, but not necessary, to design probes which hybridize only with target nucleic acids under conditions of high stringency. Only highly complementary nucleic acid hybrids form under conditions of high stringency. Accordingly, the stringency of the assay conditions determines the amount of complementarity which should exist between two nucleic acid strands in order to form a hybrid. Stringency should be chosen to maximize the difference in stability between the probe:target hybrid and the potential probe:non-target hybrids.

Probes may be designed from Egr-1 and Egr-2 nucleic acids through methods known in the art. For instance, the G+C content of the probe and the probe length can affect probe binding to its target sequence. Methods to optimize probe specificity are commonly known, such as those described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989) or Ausubel *et al.* (*Current Protocols in Molecular Biology*, Greene Publishing Co., NY, 1995).

Hybridization conditions are modified using known methods, such as those described by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989) and Ausubel *et al.* (*Current Protocols in Molecular Biology*, Greene Publishing Co., NY, 1995) as required for each probe. Hybridization of total cellular RNA or RNA enriched for polyA RNA can be accomplished in any available format. For instance, total cellular RNA or RNA enriched for polyA RNA can be affixed to a solid support and the solid support exposed to at least one probe comprising at least one, or part of one of the nucleic acids encoding Egr-1 or Egr-2 under conditions in which the probe will specifically hybridize. Alternatively, nucleic acid fragments comprising at least one, or part of one of the nucleic acids encoding Egr-1 or Egr-2 can be affixed to a solid support, such as a silicon wafer or a porous glass wafer. The wafer can then be exposed to total cellular RNA or polyA RNA from a sample under conditions in which the affixed sequences will specifically hybridize. Such glass wafers and hybridization methods are widely available, for example, those disclosed by Beattie (WO 95/11755). Such silicon wafers and hybridization methods are widely available, for example, those disclosed by Rava *et al.* (U.S. Patent No: 5,874,219). By examining for differences between the ability of a given probe to specifically hybridize to an RNA sample from an untreated cell population versus a cell population exposed to an agent to be tested, agents which up or down regulate the expression Egr-1 and/or Egr-2 are identified. Further, probes are envisaged which hybridize to the hereinabove described Egr-1 and/or Egr-2 nucleic acids if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove described polynucleotides. As used herein, the term "stringent conditions" refers to conditions such that hybridization will occur only if there is at least 95%, and preferably 97% identity between the sequences.

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Hybridization for qualitative and quantitative analysis of mRNAs may also be carried out by using a RNase Protection Assay (e.g., RPA, see Ma *et al.*, *Methods* (1996) 10: 273-278). Briefly, an expression vehicle comprising cDNA encoding the gene product and a phage specific DNA dependent RNA polymerase promoter (e.g., T7, T3 or SP6 RNA polymerase) is linearized at the 3' end of the cDNA molecule, downstream from the phage promoter, wherein such a linearized molecule is subsequently used as a template for synthesis of a labeled antisense transcript of the cDNA by *in vitro* transcription. The labeled transcript is then hybridized to a mixture of isolated RNA (e.g., total or fractionated mRNA) by incubation at 45°C overnight in a buffer comprising 80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl and 1 mM EDTA. The resulting hybrids are then digested in a buffer comprising 40 µg/ml ribonuclease A and 2 µg/ml ribonuclease. After deactivation and extraction of extraneous proteins, the samples are loaded onto urea/polyacrylamide gels for analysis.

In another assay format for identification of agents which effect the expression of the instant gene products, cells or cell lines would first be identified which express said gene products physiologically. Cell and/or cell lines so identified would be expected to comprise the necessary cellular machinery such that the fidelity of modulation of the transcriptional apparatus is maintained with regard to exogenous contact of agents with appropriate surface transduction mechanisms and/or the cytosolic cascades. Further, such cells or cell lines would be transduced or transfected with an expression vehicle (e.g., a plasmid or viral vector) construct comprising an operable non-translated 5'-promoter containing end of the structural gene encoding the instant gene products fused to one or more antigenic fragments, which are peculiar to the instant gene products, wherein said fragments are under the transcriptional control of said promoter and are expressed as polypeptides whose molecular weight can be distinguished from the naturally occurring polypeptides or may further comprise an immunologically distinct tag. Such a process is well known in the art (see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989).

Cells or cell lines transduced or transfected as outlined above would then be contacted with agents under appropriate conditions; for example, the agent comprises a pharmaceutically acceptable excipient and is contacted with cells comprised in an aqueous physiological buffer such as phosphate buffered saline (PBS) at physiological pH, Eagles

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balanced salt solution (BSS) at physiological pH; PBS or BSS comprising serum or conditioned media comprising PBS or BSS and/or serum incubated at 37°C. Said conditions may be modulated as deemed necessary by one of skill in the art. Subsequent to contacting the cells with the agent, said cells will be disrupted and the polypeptides of the disruptate are fractionated such that a polypeptide fraction is pooled and contacted with an antibody to be further processed by immunological assay (e.g., ELISA, immunoprecipitation or Western blot). The pool of proteins isolated from the "agent contacted" sample will be compared with a control sample where only the excipient is contacted with the cells and an increase or decrease in the immunologically generated signal from the "agent contacted" sample compared to the control will be used to distinguish the effectiveness of the agent.

The agents identified by the above methods can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents.

Candidate peptide agents can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems (e.g., PCR and cloning). Peptide production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included in the candidate peptide product.

Methods to Identify Agents that Modulate at Least One Activity of Egr-1 or Egr-2 Proteins

Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of the proteins encoded by Egr-1 or Egr-2. Such methods or assays may utilize any means of monitoring or detecting the desired protein(s).

In one format, the relative amounts of protein between a cell population exposed to an agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein(s) in different cell populations. Cell lines or populations are

exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe.

Antibodies directed against Egr-1 and Egr-2 encoded polypeptides are well known in the art (see Waters *et al.*, *Oncogene* (1990) 5(5):699-674 and Skerka *et al.*, *Immunobiology* (1997) 198(1-3):179-191, respectively) and such antibodies may be used as probes. Further, antibodies against Egr-1 and Egr-2 proteins may be prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides, polypeptides or proteins if they are of sufficient length, or, if desired, or if required to enhance

immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a Cys residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier.

Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or modifications which effect

immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten, polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab' or F(ab')₂ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the gene products can also be produced in the context of chimeras with multiple species origin.

In an alternative format, a specific activity of a protein may be assayed, such as the ability of the protein to bind to a substrate such as a GSG consensus comprising nucleic acid or by transactivation of an appropriate gene product (Cibelli *et al.*, *Eur J Biochem* (1996) 237(1):311-7). Cell lines or populations are exposed under appropriate conditions to the agent to be tested. Agents which modulate the binding activity of the protein are identified by assaying the binding activity of the protein from the exposed cell line or population and a control, unexposed cell line or population, thereby identifying agents which modulate the binding activity of the protein.

Binding assays to measure the ability of the agent to modulate the binding activity of an Egr-1 or Egr-2 protein are widely available such as the assays disclosed by Morris *et al.*, *Oncogene* (1991) 6(12):2339-48 and Cibelli *et al.*, *Eur J Biochem* (1996) 237(1):311-7.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the an Egr-1 or Egr-2 protein alone or with its associated substrates, binding partners, etc.

An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Another class of agents of the present invention are antibodies immunoreactive with critical positions of Egr-1 or Egr-2 proteins.

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Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies. Antibodies can also be generated by expression of proteins in a variety of culture systems (bacterial, yeast, or mammalian systems) and any portions of the predicted amino acid sequences can be used as an immunogen.

EXAMPLES

Example 1

Identification of Differentially Expressed Ischemic Heart mRNA

Heart tissue was obtained from subjects exhibiting post-ischemic cardiomyopathy and their age- and sex-matched controls. Tissues were obtained from cardiocotomized patients or by biopsy. Biopsied materials from heart tissues were obtained by standard procedures associated with heart transplantation and follow-up and during catheterization after heart attack (Gout *et al.*, *N Engl J Med* (1990) 322:383-388; Beck *et al.*, *West J Med* (1991) 155(3): 293; Thibault G.E., *N Engl J Med* (1992) 327:714-717; MacLellan *et al.*, *Pediatrics* (1995) 96:122-125; and Book *et al.*, *Cathet Cardiovasc Diagn* (1998) 45(2):167-169).

Total cellular RNA was prepared from the heart tissue described above as well as from control, non-ischemic heart tissue using the procedure of Newburger *et al.* (1981) *J Biol. Chem.* 266(24):16171-7 and Newburger *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5215-5219.

Synthesis of cDNA was performed as previously described by Prashar *et al.* in WO 97/05286 and in Prashar *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:659-663. Briefly, cDNA was synthesized according to the protocol described in the GIBCO/BRL kit for cDNA synthesis. The reaction mixture for first-strand synthesis included 6 µg of total RNA, and 200 ng of a mixture of anchored oligo(dT) primers degenerate at 3'.

(ACGTAATACGACTACTATAGGGCGAATTGGGTGCACTTTT) where n1=A/C or G) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was incubated at 65°C for 5 mins, chilled on ice and the process repeated. Alternatively, the reaction mixture may include 10µg of total RNA, and 2

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pmol of 1 of the 2-base anchored oligo(dT) primers a heel such as RP5.0 (CTCTCAAGGATCTTACCGCTT_{n1}AT), or RP9.2 (TAATACCGCGGCACATAGCAT_{n2}CG), or RP6.0 (TAATACCGCGGCACATAGCAT_{n2}CG), along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was then layered with mineral

oil and incubated at 65°C for 7 min followed by 50°C for another 7 min. At this stage, 2µl of SUPERScript REVERSE TRANSCRIPTASE® (200 units/µl; GIBCO/BRL) was added quickly and mixed, and the reaction continued for 1 hr at 45-50°C. Second-strand synthesis was performed at 16°C for 2 hr. At the end of the reaction, the cDNAs were precipitated with ethanol and the yield of cDNA was calculated. In our experiments, ~200 ng of cDNA was obtained from 10µg of total RNA.

The adapter oligonucleotide sequences were phosphorylated at 5' end using A1 (TAGCGTCCGGCGGACAGGAGCGGCCAG) and A2 (GATCTGCGCCCTCGGCTGTCTCGGCCG). One microgram of

oligonucleotide A2 was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After phosphorylation, PNK was heat denatured, and 1µg of the oligonucleotide A1 was added along with 10% annealing buffer (1 M NaCl/100 mM Tris-HCl, pH8.0/10 mM EDTA, pH8.0) in a final vol of 20 µl. This mixture was then heated at 65°C for 10 min followed by slow cooling to room temperature for 30 min, resulting in formation of the Y adapter at a final concentration of 100 ng/µl. About 20 ng of the cDNA was digested with 4 units of Bgl II in a final vol of 10 µl for 30 min at 37°C. Two microliters (4 ng of digested cDNA) of this reaction mixture was then used for ligation to 100 ng (=50-fold) of the Y-shaped adapter in a final vol of 5µl for 16 hr at 15°C. After ligation, the reaction mixture was diluted with water to a final vol of 80 µl (adapter ligated cDNA concentration, ~50 pg/µl) and heated at 65°C for 10 min to denature T4 DNA ligase, and 2-µl aliquots (with ~100 pg of cDNA) were used for PCR.

The following sets of primers were used for PCR amplification of the adapter ligated 3' end cDNAs:

TGAAGCCGAGACGTCGCTGGT_{n1}, n2 (wherein n1, n2 = AA, AC, AG AT CA CC CG CT GA GC GG and GT) as the 3' primer with A1 as the 5' primer or alternatively

RP 5.0, RP 6.0, or RP 9.2 used as 3' primers with primer A1.1 serving as the 5' primer. To detect the PCR products on the display gel, 24 pmol of oligonucleotide A1 or A1.1 was 5'-end-labeled using 15 µl of [γ -³²P]ATP (Amersham; 3000 Ci/mmol) and PNK in a final volume of 20 µl for 30 min at 37°C. After heat denaturing PNK at 65°C for 20 min, the labeled oligonucleotide was diluted to a final concentration of 2 µM in 80 µl with unlabeled oligonucleotide A1.1. The PCR mixture (20 µl) consisted of 2 µl (=100 pg) of the template, 2 µl of 10× PCR buffer (100 mM Tris HCl, pH 8.3/500 mM KCl), 2 µl of 15 mM MgCl₂ to yield 1.5 mM final Mg²⁺ concentration optimum in the reaction mixture, 200 µM dNTPs, 200 nM each 5' and 3' PCR primers, and 1 unit of AmpliTaq Gold®. Primers and dNTPs were added after preheating the reaction mixture containing the rest of the components at 85°C. This "hot start" PCR was done to avoid artefactual amplification arising out of arbitrary annealing of PCR primers at lower temperatures during transition from room temperature to 94°C in the first PCR cycle. PCR consisted of 5 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 60 sec followed by 25 cycles of 94°C for 30 sec, 60°C for 2 min, and 72°C for 60 sec. A higher number of cycles resulted in smeary gel patterns. PCR products (2.5 µl) were analyzed on 6% polyacrylamide sequencing gel. For double or multiple digestion following adapter ligation, 13.2 µl of the ligated cDNA sample was digested with a secondary restriction enzyme(s) in a final vol of 20 µl. From this solution, 3 µl was used as a template for PCR. This template vol of 3 µl carried 100 pg of the cDNA and 10 mM MgCl₂ (from the 10× enzyme buffer), which diluted to the optimum of 1.5 mM in the final PCR vol of 20 µl. Since Mg²⁺ comes from the restriction enzyme buffer, it was not included in the reaction mixture when amplifying secondarily cut cDNA. Individual cDNA fragments corresponding to mRNA species were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Bands were extracted from the display gels as described by Liang *et al.* (1995 *Curr. Opin. Immunol.* 7:274-280), reamplified using the 5' and 3' primers, and subcloned into PCR-Script with high efficiency using the PCR-Script cloning kit from Stratagene. Plasmids were sequenced by cycle sequencing on an ABI automated sequencer. Alternatively, bands were extracted (cored) from the display gels, PCR amplified and sequenced directly without subcloning.

Example 2

A Northern blot analysis was performed using a probe derived from SEQ ID Nos. 1 and 3 with hybridization conditions as described by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989). PCR expression analysis is also performed using primers derived from EGR-1 or EGR-2 using AmpliTaq Gold PCR® amplification kits (Perkin Elmer).

Real time PCR detection is accomplished by the use of the ABI PRISM 7700 (Applied Biosystems) Sequence Detection System. The 7700 will measure the fluorescence intensity of the sample each cycle and is able to detect the presence of specific amplicons within the PCR reaction. Each sample is assayed for the level of GAPDH and clones 1084 (Egr-1) and 1084A (Egr-2). GAPDH detection is performed using Perkin Elmer part #402869 according to the manufacturer's directions. Primers are designed for clones 1084 (Egr-1) and 1084A (Egr-2) using Primer Express, a program developed by PE to efficiently find primers and probes for specific sequences. These primers are used in conjunction with SYBR green (Molecular Probes), a nonspecific double stranded DNA dye, to measure the expression level of clones 1084 and/or 1084A, which is normalized to the GAPDH level in each sample.

Method of Screening for Modulators of Myocardial Egr1 and/or Egr-2 Expression

Using human epithelial cells, a shear-stress of 1.5 N/m² is applied to cells in culture according to the method of Houston *et al.* (*Arterioscler Thromb Vasc Biol* (1999) 19(2):281-289). At specific time points during applied stress, candidate agents and diluent (i.e., carrier minus agent; control) are contacted with human epithelial cells. Cells are removed and lysed in an appropriate buffer for isolation of total and/or messenger RNA in a similar fashion as described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989). Isolated nucleic acids are then assayed by a transcriptional profiling means

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to determine whether the candidate agent modulates the induction of Egr-1 and/or Egr-2.

Agents which up- or down-regulate the expression of either one or both transcriptional factors are then designated as modulators of Egr-1 and/or Egr-2.

Example 4

Method of Screening for Modulators of Myocardial Egr-1 and/or Egr-2 Expression

Using Passive Stretch of Cardiomyocytes

Using cultured myocytes on silicone membranes, cells are passively stretched according to the method of Yamazaki *et al.* (*J Mol Cell Cardiol* (1995) 27(1):133-140). At specific time points during applied stress, candidate agents and diluent (i.e., carrier minus agent; control) are contacted with human epithelial cells. Control and test cells are removed and lysed in an appropriate buffer for isolation of total and/or messenger RNA in a similar fashion as described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989). Isolated nucleic acids are then assayed by a transcriptional profiling assay to determine whether the candidate agent modulates the induction of Egr-1 and/or Egr-2. Agents which up- or down-regulate the expression of either one or both transcriptional factors will then be designated as modulators of Egr-1 and/or Egr-2.

Agents which modulate the expression of Egr-1 and/or Egr-2 are then designated as modulators of Egr-1 and/or Egr-2.

Method of Screening for Modulators of Myocardial Egr-1 and/or Egr-2 Expression

Using an Animal Model for Occlusion and Reproduction of the Heart

Animal models for occlusion of the heart are well documented (Solomon *et al.*, *J Am Coll Cardiol* (1999) 33(3): 854-856 and Kirma *et al.*, *Jpn Circ J* (1998) 62(4):294-298). For example, pigs are used wherein regional ischemia is produced in control and candidate agent treated animals by partially occluding (ligating) the left anterior descending coronary artery. After obtaining the baseline values for evaluation of heart rate, transmural flow and blood pressure, agents are administered to the animals (including carrier-only for controls) and at various time points and/or after administration of various concentrations of candidate agents using a single time point, post occlusion, the hearts of the animals are removed for isolation of nucleic acids by standard methods as described in Sambrook *et al.* (*Molecular Cloning: A*

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Laboratory Manual, Cold Spring Harbor Press, NY, 1989). Isolated nucleic acids are then assayed by a transcriptional profiling assay to determine whether the candidate agent modulates the induction of Egr-1 and/or Egr-2. Agents which up- or down-regulate of either one or both transcriptional factors will then be designated as modulators of Egr-1 and/or Egr-2.

5 2.

1. agent

Isolation of Forensic Method of Determining Cause of Death Due to CAD

3. Samples are obtained from the post mortem heart tissue of expired patients where cause of death is suspected to be from CAD (test sample) and are compared to postmortem tissue isolated from aged and sex matched controls where death by heart disease has been excluded. Tissues are prepared for isolation of nucleic acids by standard methods as

described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, 1989). Isolated nucleic acids are then assayed by a transcriptional profiling assay to determine whether induction of Egr-1 and/or Egr-2 has occurred. The observation of induced Egr-1 and/or Egr-2 in the test sample is diagnostic of CAD as the cause of death.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

We claim:

1. A method of diagnosing coronary artery disease (CAD) in a patient comprising the steps of:

- a) obtaining a nucleic acid sample from the cardiac muscle of a patient;
- b) assaying for the induction of Egr-1 or Egr-2 mRNA in said nucleic acid sample, wherein the induction of Egr-1 or Egr-2 mRNA is an indication of CAD.

2. The method of claim 1, wherein CAD is chronic diffuse myocardial ischemia.

3. The method of claim 1, wherein the nucleic acid is from heart biopsy material obtained during catheterization.

4. The method claim 1, wherein the nucleic acid is from heart biopsy material obtained during transplant typing.

5. The method of claim 1, wherein the assay comprises a transcriptional profiling (TP) assay.

6. The method of claim 5, wherein said TP assay is selected from the group consisting of RNAse protection assays, RT-PCR, Northern blot and TAQMAN assay.

7. A method of screening for agents which modulate Egr-1 gene expression comprising the steps:

- a) incubating cells under conditions which simulate heart stress or cardiac ischemia in the presence or absence of an agent;
- b) determining whether Egr-1 is up- or down-regulated in the presence or absence of the agent, wherein up- or down-regulation of Egr-1 identifies the agent as a modulator.

8. A method of screening for agents which modulate Egr-2 gene expression comprising the steps:

- a) incubating cells under conditions which simulate heart stress or cardiac ischemia in the presence or absence of an agent;
- b) determining whether Egr-2 is up- or down regulated in the presence or absence of the agent, wherein up- or down-regulation of Egr-2 identifies the agent as a modulator.

9. The method of claim 7, wherein the incubation conditions comprise shear stress.

10. The method of claim 7, wherein the incubation conditions comprise passive stretch of myocytes.

11. The method of claim 8, wherein the incubation conditions comprise shear stress.

12. The method of claim 8, wherein the incubation conditions comprise passive stretch of myocytes.

13. A method of screening for agents which modulate Egr-1 gene expression comprising the steps:

- a) occluding blood vessels leading to the heart of test and control animals to produce conditions which simulate cardiac ischemia and
- b) determining whether Egr-1 is up- or down-regulated in the presence or absence of an administered agent, wherein up or down regulation of Egr-1 identifies the agent as a modulator.

14. A method of screening for agents which modulate Egr-2 gene expression comprising the steps:

- a) occluding blood vessels leading to the heart of test and control animals to produce conditions which simulate cardiac ischemia and

b) determining whether Egr-2 is up- or down-regulated in the presence or absence of an administered agent, wherein up- or down-regulation of Egr-2 identifies the agent as a modulator.

15. A forensic method of determining cause of death due to coronary artery disease (CAD) comprising the steps:

a) obtaining a postmortem sample from the cardiac muscle of a patient, wherein CAD is suspected as the cause of death;

b) assaying for the induction of Egr-1 mRNA in said nucleic acid sample, wherein the induction of Egr-1 mRNA is an indication of death due to CAD.

16. A forensic method of determining cause of death where to coronary artery disease (CAD) comprising the steps:

a) obtaining a postmortem sample from the cardiac muscle of a patient, wherein CAD is suspected as the cause of death;

b) assaying for the induction of Egr-2 mRNA in said nucleic acid sample,

17. wherein the induction of Egr-2 mRNA is an indication of death due to CAD.

17. A forensic method of determining cause of death due to coronary artery disease (CAD) comprising the steps:

a) obtaining a postmortem sample from the cardiac muscle of a patient, wherein CAD is suspected as the cause of death;

b) assaying for the induction of Egr-1 and Egr-2 mRNA in said nucleic acid sample, wherein the induction of Egr-1 and Egr-2 mRNA is an indication of death due to CAD.

FIG. 1A

EGR1 (Sequence 1084 Q-PCR Results)
READS regulation = Up



FIG. 1B

EGR1 (Sequence 1084 Q-PCR Results)
READS regulation = Up

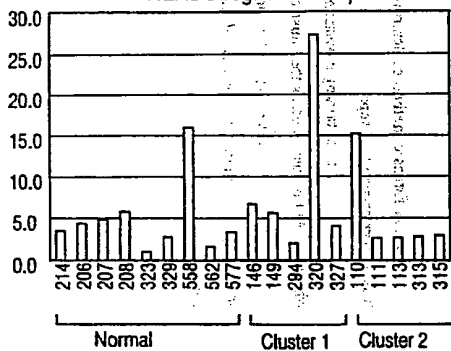


FIG. 1C

EGR1 (Sequence 1084 Q-PCR Results)
READS regulation = Up

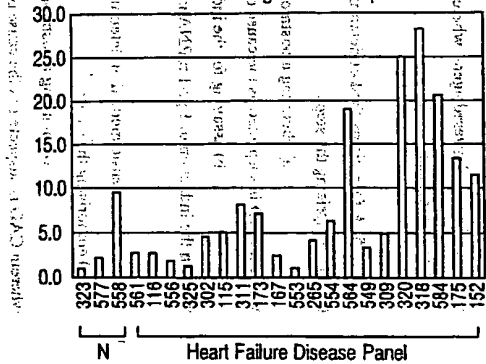


FIG. 2A

EGR2 (Sequence 1084A)
Q-PCR Results

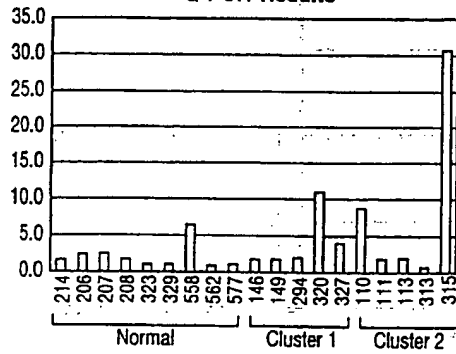


FIG. 2B

EGR2 (Sequence 1084A)
Q-PCR Results

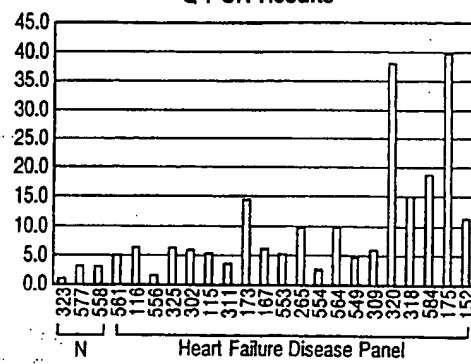
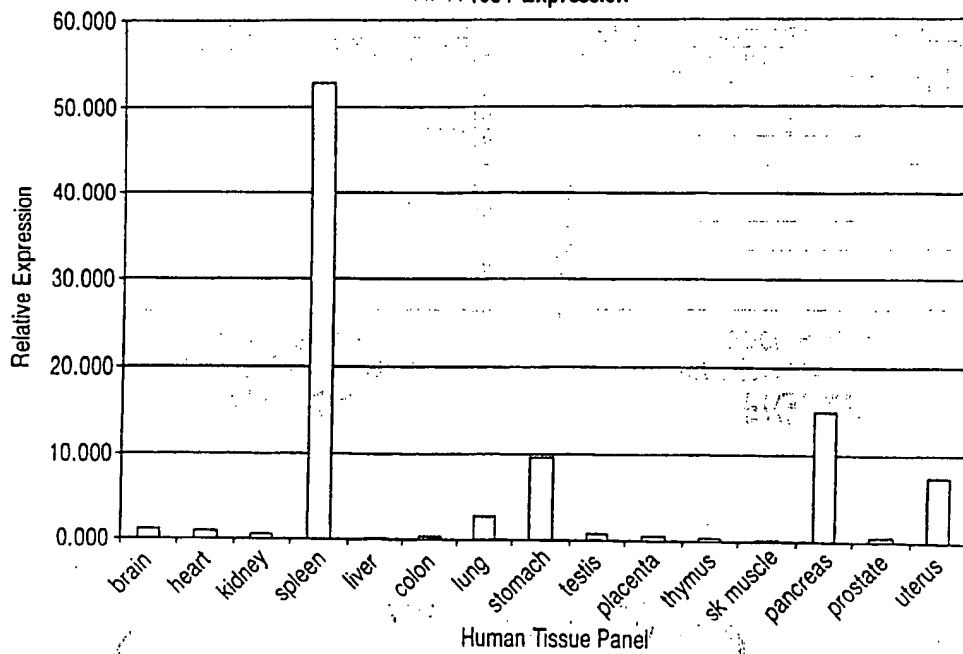
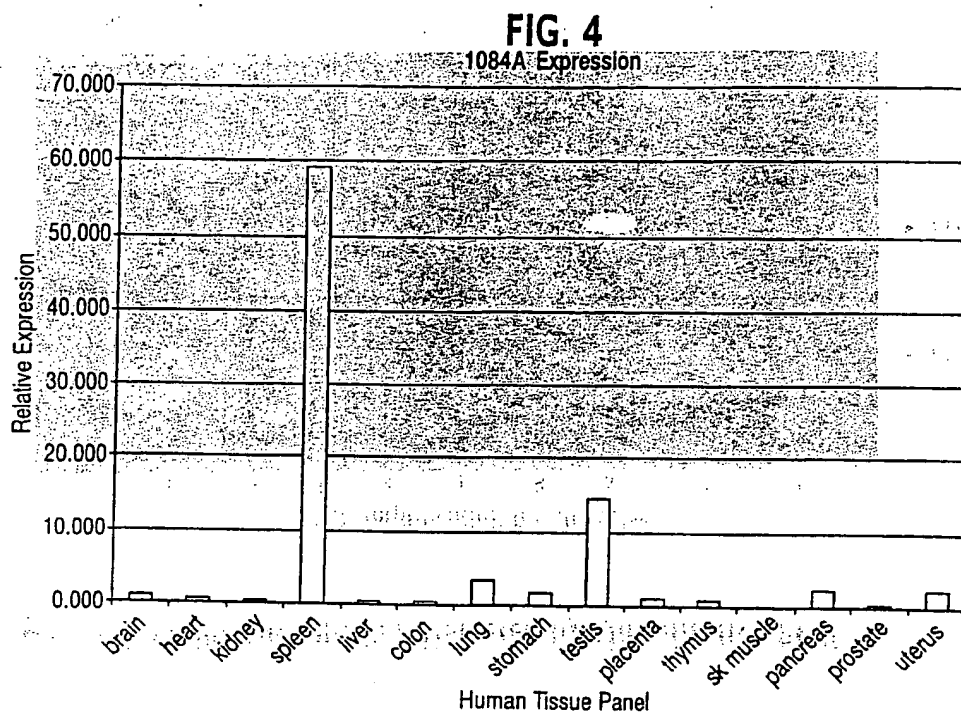
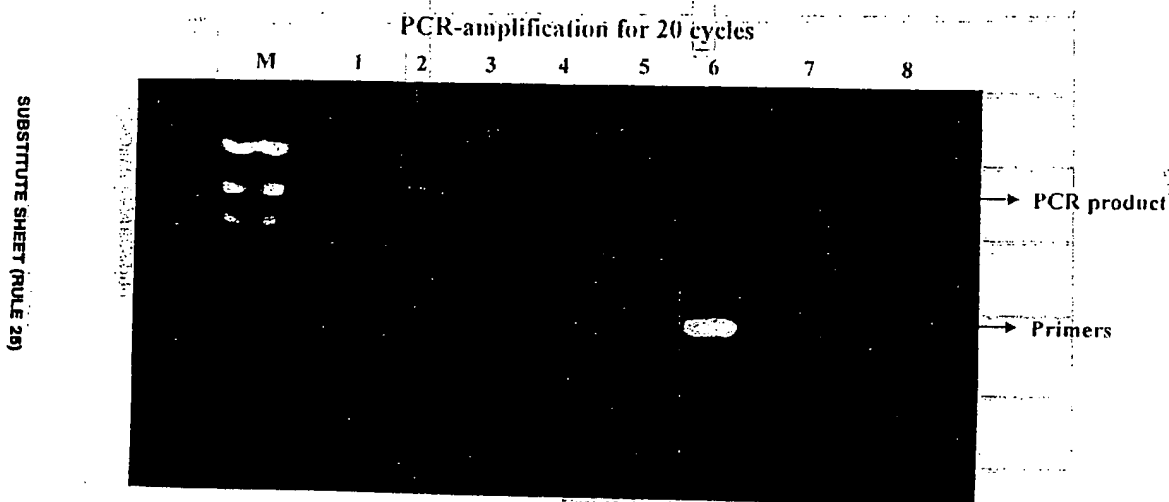


FIG. 3

1084 Expression



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**FIG. 5** EGR1 Tissue Distribution Detected by Quantitative PCR

M= DNA marker

PCR templates: 1. Brain; 2. Heart; 3. S. muscle; 4. Kidney; 5. Leukocytes; 6. Liver; 7. Lung;
8. Spleen.

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Thr Gly Glu Lys Pro Phe Ala Cys Asp Ile Cys Gly Arg Lys Phe Ala 335
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ggg aag agg tcy ttg gat ctc cca tat ccc agc agc ttt gct ccc gtc 96
Glu Lys Arg Ser Leu Asp Leu Pro Tyr Pro Ser Ser Phe Ala Pro Val 20
25 30

tct gca cct aga aac cag acc ttc act tac atg ggc aag ttc tcc att 144
Ser Ala Pro Arg Asn Gln Thr Thr Tyr Met Gly Lys Phe Ser Ile 45
35 40 45
gac cca, cag tac cct ggt gcc agc tgc tac cca gaa ggc ata atc aat. 192
Asp Pro Gln Tyr Pro Gly Ala Ser Cys Tyr Pro Gln Gly Ile Ile Asn 50
55 60
att gtg agt gca ggc atc ttg caa ggg gtc act tcc cca gct tca acc 240
Ile Val Ser Ala Gly Ile Leu Gln Gly Val Thr Ser Pro Ala Ser Thr 65
70 75 80
aca ggc tca tcc agc gtc acc tct gcc tcc ccc aac cca ctg gcc aca 288
Thr Ala Ser Ser Val Thr Ser Ala Ser Pro Asn Pro Leu Ala Thr 85
90 95
gga ccc ctg ggt gtg tgc acc atg tcc cag acc cag cct gac ctg gac. 336
Gly Pro Leu Gly Val Cys Thr Met Ser Gln Thr Gln Pro Asp Leu Asp 100
105 110
cac ctg tac tct, ccg cca ccg cct cct cct cct tat tct ggc tgt gca 384
His Leu Tyr Ser Pro Pro Pro Pro Pro Pro Tyr Ser Gly Cys Ala 115
120 125 130
gga gac ctc tac cag gac cct tct gcg ttc ctg tca gca gcc acc acc 432
Gly Asp Leu Tyr Gln Asp Pro Ser Ala Phe Leu Ser Ala Ala Thr Thr 135
140
tcc acc tct tcc tct, ctg gcc tac cca, cca cct cct, tcc tat, cca tcc 480
Ser Thr Ser Ser Ser Leu Ala, Tyr Pro Pro Pro Pro Ser Tyr Pro Ser 145
150 155 160
ccc aag cca, gcc acc ggc cca ggc ctc ttc cca atg atc cca gac tat 528
Pro Lys Pro Ala Thr Asp Pro Gly Leu Phe Pro Met Ile Pro Asp Tyr 165
170 175
cct gga ttc ttt cca tct cag tgc cag aga gac cta cat ggt aca gct 576
Pro Gly Phe Phe Pro Ser Gln Cys Gln Arg Asp Leu His Gly Thr Ala 180
185 190
ggc cca gac cgt aag ccc ttt ccc tgc cca ctg gac acc ctg cgg gtg 624
Gly Pro Asp Arg Lys Pro Phe Pro Cys Pro Leu Asp Thr Leu Arg Val 195
200 205
ccc cct cca ctc act cca ctc tct aca atc cgt aac ttt acc ctg ggg 672
Pro Pro Pro Leu Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 210
215 220
ggc ccc agt gct ggg atg acc gga cca, ggg gcc agt gga ggc agc gag 720
Gly Pro Ser Ala Gly Met Thr Gly Pro Gly Ala Ser Gly Gly Ser Gly. 225
230 235 240
gga cca cgg ctg cct ggt agc agc tca, gca gca gca gcc gcc gcc. 768
Gly Pro Arg Leu Pro Gly Ser Ser Ser Ala Ala Ala Ala Ala Ala Ala 245
250 255

[illegible]

Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Asp	Pro	Gln	Tyr	Pro	Gly	Ala	Ser	Cys	Tyr	Pro	Glu	Gly	Ile	Ile	Asn	150	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345</	

2019年12月31日

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International application No.
PCT/US00/18923

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim
Y	DEINDL ¹ et al. Gene expression after short periods of coronary occlusion. Molecular and Cellular Biochemistry. September 1998, Vol. 186, No. 1 & 2, pages 43-51, especially pages 44-45.	13
A	AEBERT et al. Expression of immediate early genes after cardioplegic arrest and reperfusion. Annals of Thoracic Surgery. June 1997, Vol. 63, No. 6, pages 1669-1675.	1-6, 15, 17
A	US 5,726,288 A (CALL ET AL) 10 March 1998 (10/3/98), see entire document.	1-17

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INTERNATIONAL SEARCH REPORT

1890

International application No.
PCT/US00/18923

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used)

USPT, DWPI

coronary, myocardial, cardiovascular, ischemia, disease, disorder, attack, infarction, arrest, chronic diffuse myocardial

[illegible][illegible]

THESE THINGS ARE NOT THE SAME AS THE OTHERS

2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 2679, 2680, 2681, 2682, 2683, 26

Table 1. *Continued*

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	男	女
一、二、三、四、五、六、七、八、九、十、十一、十二、十三、十四、十五、十六、十七、十八、十九、二十、二十一、二十二、二十三、二十四、二十五、二十六、二十七、二十八、二十九、三十、三十一、三十二、三十三、三十四、三十五、三十六、三十七、三十八、三十九、四十、四十一、四十二、四十三、四十四、四十五、四十六、四十七、四十八、四十九、五十、五十一、五十二、五十三、五十四、五十五、五十六、五十七、五十八、五十九、六十、六十一、六十二、六十三、六十四、六十五、六十六、六十七、六十八、六十九、七十、七十一、七十二、七十三、七十四、七十五、七十六、七十七、七十八、七十九、八十、八十一、八十二、八十三、八十四、八十五、八十六、八十七、八十八、八十九、九十、九十一、九十二、九十三、九十四、九十五、九十六、九十七、九十八、九十九、一百	一、二、三、四、五、六、七、八、九、十、十一、十二、十三、十四、十五、十六、十七、十八、十九、二十、二十一、二十二、二十三、二十四、二十五、二十六、二十七、二十八、二十九、三十、三十一、三十二、三十三、三十四、三十五、三十六、三十七、三十八、三十九、四十、四十一、四十二、四十三、四十四、四十五、四十六、四十七、四十八、四十九、五十、五十一、五十二、五十三、五十四、五十五、五十六、五十七、五十八、五十九、六十、六十一、六十二、六十三、六十四、六十五、六十六、六十七、六十八、六十九、七十、七十一、七十二、七十三、七十四、七十五、七十六、七十七、七十八、七十九、八十、八十一、八十二、八十三、八十四、八十五、八十六、八十七、八十八、八十九、九十、九十一、九十二、九十三、九十四、九十五、九十六、九十七、九十八、九十九、一百	

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